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We have previously isolated an intact, stable, and fully functional multiprotein DNA replication complex (designated the DNA synthesome) from a variety of non-malignant and malignant tumor cells and tissues including breast cancer cells. We have also shown that poly(ADP-ribose) polymerase (PARP) is among the components of the DNA synthesome. The transformation of a non-malignant human cell to a malignant stat is accompanied by a significant alteration in the mobility of specific components of the DNA synthesome, (such as PCNA) following 2D-PAGE analysis of the DNA synthesome, together with a 4-6-fold decrease in the replication fidelity of the replication complex. In order to establish whether the malignant transformation process is accompanied by an alteration in PARP, we purified PARP from malignant and non-malignant breast cells using phosphocellulose and hydroxylapatite chromatography. PARP was enriched during its purification from these cells. We have also found that PARP isolated from malignant breast cells exhibits a different migration pattern, when analyzed by 2D-PAGE, compared to the enzyme isolated from non-malignant breast cells; indicating that this enzyme may be modified in breast cancer cells. Mass spectrometric analyses and physical characteristics determination of PARP from malignant and non-malignant breast cells are underway in order to explain how PARP modification may contribute to the observed decrease in replication fidelity in breast cancer cells.

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INTRODUCTION:

The genetic damage which accompanies the development and progression of breast cancer has been linked to defects in the DNA replication and repair processes in these cells. We have previously isolated an intact, stable and fully functional multiprotein DNA replication complex (designated the DNA synthesome) from a variety of non-malignant as well as malignant tumor cells and tissues including breast cancer cells. All of the components necessary for DNA replication, including poly(ADP-ribose) polymerase (PARP) have been detected in the DNA synthesome. We have shown that the malignant breast cell DNA synthesome exhibits a 4-6-fold decrease in replication fidelity relative to the non-malignant breast cell DNA synthesome (Sekowski et al., 1998). In addition, the transformation of a non-malignant human breast epithelial cells to a malignant state is accompanied by a significant alteration in the mobility of specific protein components of the DNA synthesome (such as proliferating cell nuclear antigen; PCNA) following two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of the replication complex (Bechtel et al., 1998). PARP is a nuclear enzyme of a molecular weight of 116,000. It plays an important role in DNA replication, repair, and recombination. It absolutely requires DNA single-strand or double-strand breaks for its enzymatic activity. This enzyme, once activated, catalyzes the transfer of ADP-ribose unit from nicotinamide adenine dinucleotide (NAD) to nuclear proteins such as histone and PARP itself. It has been shown that at least fifteen components of the DNA synthesome are poly(ADP-ribosylated), suggesting that PARP may play a regulatory role in controlling the activity and the fidelity of the DNA synthesome (Simbulan-Rosenthal et al., 1996 and 1998). The unique form of PCNA found exclusively in malignant breast cells lacks the poly(ADP-ribose) modification which is found in the non-malignant form of the protein. The goal of this project is to establish a link between the differences in PARP activity and the alteration(s) in structure exhibited by this protein in both malignant and non-malignant breast cells.

BODY:

I- PARP was confirmed to be one of the Components of the DNA Synthesome:

The DNA synthesome has been isolated and purified from breast cancer MDA MB-468 cells using a series of steps, which include centrifugation, polyethylene glycol precipitation, ion exchange chromatography, and density gradient sedimentation (Malkas et al., 1990; Applegren et al., 1995; Coll et al., 1996; Lin et al., 1997) (Figure 1A). P4 fraction (5 ml) containing approximately 30 mg of protein was loaded onto a Bio-Rad Q5 column pre-equilibrated with a buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 0.1% glycerol, and 1 mM EDTA. The column was then washed with the same buffer and eluted using a gradient of 50-500 mM KCl. One-milliliter fractions were collected. The eluted fractions were then dialyzed against a buffer containing 20 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT and aliquots of each fraction were stored at –80°C.

The P4 and Q-sepharose peaks were resolved through 10% denaturing polyacrylamide gel electrophoresis (SDS-PAGE). Western blot analysis was performed using anti-human PARP antibody (BD PharMingen). PARP was found to exclusively co-purify with the DNA synthesome (Figure 1B).

Mass spectrometric analysis of the DNA synthesome enriched protein fractions also indicated that PARP is a part of the replication complex core. Each of the protein bands from SDS-PAGE was cut and analyzed by MALDI and Q-TOF mass spectrometry. It has been shown that PARP, PCNA, replication protein A (RPA), replication protein C (RFC), DNA polymerase α, DNA methyltransferase, and FEN1 are components of the replication complex core (Dr. Suhua Han's 2001 Annual Report).

II- PARP Purification using Phosphocellulose and Hydroxylapatite Chromatography:

In order to define the kinetic and physical characteristics of PARP, we conducted chromatographic procedures to purify that protein from malignant and non-malignant breast cells. We initiated experiments to purify PARP from the malignant MDA MB-468 and non-malignant MCF-10A cells according to the methods of Ushiro et al. (1987), Jump and Smulson (1980), and D'Amours et al. (1997). The purification procedures involved phosphocellulose followed by hydroxylapatite chromatography.

1- Phosphocellulose Purification of PARP:

The first step of PARP purification involved the use of a phosphocellulose column that dissociated the synthesome and resolved PARP from other proteins.

Cellulose phosphate (Sigma) was prepared according to the following protocol:

1- The resin was suspended in 5 volumes of distilled water overnight. It was stirred and allowed to settle for 45 minutes. The settled volume was measured and this was considered the column volume (CV) required to measure the volumes of the washing solution.
2- The resin was suspended in 5 CV of 0.05 M NaOH containing 0.5 M NaCl for 10 minutes and the slurry was poured into a sintered glass funnel while applying gentle suction.
3- The slurry was washed with 5 CV of distilled deionized water.
4- The resin was suspended with 5 CV of 0.75 N HCl for 10 minutes and was poured back into the funnel. This was followed by passing fresh 0.75 N HCl through the bed.
5- The resin was washed with distilled deionized water using at least 9 CV until the effluent shows a pH=5.
6- The resin was packed into a column and equilibrated with **buffer A** containing 20 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 1.5 mM $MgCl_2$, and 1 mM DTT.

10 grams of MDA MB-468 and MCF-10A cells were fractionated to the clarified nuclear extract (NE) according to our published protocol (Coll et al., 1996) (Figure 1A). The presence of PARP in the nuclear fraction (NE) was detected by SDS-PAGE (10%) and immunoblot analyses of 50 μg proteins of the homogenate (H), cytosolic (S1), and nuclear (NE) fractions. Most of PARP protein was detected in the NE fraction (Figure 2).

The NE fraction was dialyzed against **buffer A** and applied onto the phosphocellulose column equilibrated with the same buffer. PARP was eluted by a stepwise increase in NaCl concentration from 0.3 M to 0.7 M. 50 μg of NE protein fraction and 1 μg of the 0.3-0.7 M NaCl eluted fractions were resolved by 10% SDS-PAGE and PARP was detected by Western blot analysis using anti-human PARP antibody. It has been found that most of the enzyme was eluted with 0.5 M NaCl (Figure 3). PARP has been greatly enriched during this step of purification.

2- Concentration of PARP Protein Present in the Phosphocellulose Peak:

The phosphocellulose peak fractions enriched with PARP activity were concentrated using Centrifugal Filter Devices (Millipore) with a molecular weight cut-off of 50,000. The phosphocellulose peak was applied into the sample reservoir and concentrated according the instruction manual. 5 μl of concentrated fraction was then analyzed by 10% SDS-PAGE and PARP was detected by Western blot analysis using anti-human PARP antibody (Figure 4). PARP was further enriched after this step of purification.

3- Hydroxylapatite Purification of PARP:

Purification of PARP using hydroxylapatite chromatography was carried out according the method of Jump and Smulson (1980).

Hydroxylapatite matrix (Fluka BioChemika) was prepared according to the following procedures:

1- Approximately one part hydroxylapatite matrix was added to six parts loading buffer (10 mM potassium phosphate buffer, pH 6.8) and resuspend by gentle swirling.

2- The slurry was allowed to settle for a minimum of 20 minutes and the fines were decanted.

3- The loading buffer was added to the settled bed and mixed gently by swirling. The matrix was allowed to settle.

4- After the matrix settled to give a sharp dividing line between the bed and the clear buffer above, it was resuspended by swirling and poured into the column.

NE fraction (MDA MB-468) was dialyzed against **buffer B,** containing 20 mM Tris-HCl pH 7.5, 10 mM potassium phosphate buffer, 1.5 mM $MgCl_2$, and 1 mM DTT, and applied onto the hydroxylapatite column equilibrated with the same buffer. PARP was eluted with a stepwise increase in potassium phosphate concentration (10-100 mM, pH 6.8). NE, flow throw (FT), and the potassium phosphate eluted fractions were resolved by 10% SDS-PAGE and PARP was detected by Western blot analysis. Most PARP protein was detected in the 500 mM potassium phosphate eluted fraction (Figure 5).

4- Purification of PARP using a Combination of Phosphocellulose and Hydroxylapatite Chromatography:

After establishing the conditions for hydroxylapatite purification of PARP, we combined both the phosphocellulose and hydroxylapatite chromatography techniques and PARP has been purified according the following procedures:

1- NE fractions from either MDA MB-468 or MCF-10A cells were prepared according to Coll et al. (1996). They were dialyzed against **buffer A** and applied onto phosphocellulose column equilibrated with the same buffer. PARP was eluted with increasing concentration of NaCl (0.3-0.7 M).

2- The phosphocellulose peaks (fractions eluted with 0.5 M NaCl, Figure 3) were dialyzed against **buffer B** and applied onto hydroxylapatite column equilibrated with the same buffer.

3- PARP was eluted with increasing concentrations of potassium phosphate buffer (25-1000 mM, pH 6.8).

4- NE, phosphocellulose peak, FT, and the potassium phosphate eluted fractions were resolved through 10% SDS-PAGE and PARP was detected by Western blot analysis.

Figure 6 shows that bands corresponding to PARP have been detected in the NE fraction and the phosphocellulose peak (0.5 M NaCl eluted fraction). A very faint

PARP band was also detected in the hydroxylapatite fraction eluted with 500 mM potassium phosphate buffer.

In order to clearly detect PARP in the hydroxylapatite-eluted fractions, we concentrated these fractions using a speed-vac. The concentrated factions together with the NE and the phosphocellulose peak were resolved by 10% SDS-PAGE and PARP was detected by Western blot analysis (Figure 7). In addition to the detection of PARP bands in the NE and the phosphocellulose peak, a clear band appeared in the 500 mM potassium phosphate eluate. This band corresponds to a higher molecular weight protein; however, because of the high concentration of potassium phosphate in the eluted fractions, this salt precipitated after concentrating the fraction using the speed-vac. The high salt concentration slowed the migration of the sample through the gel especially the samples eluted with 100-1000 mM of potassium phosphate.

In order to make sure that this band corresponds to PARP, we had to remove the salt from the hydroxylapatite-eluted fractions. We tried to clean the hydroxylapatite peak using Bio-Spin 30 desalting columns (Bio-Rad). These columns contain Bio-Gel P-30 polyacrylamide gel suspended in 1 ml Tris buffer (10 mM Tris-HCl, pH 7.4) (BioRad Catalog number 732-6231).

In order to remove potassium phosphate from the hydroxylapatite peak, we applied the following procedures:

1- The desalting columns were inverted several times to resuspend the settled gel and remove any bubbles.

2- The tip was snapped off and the column was placed in a 2.0 ml microcentrifuge tube. The cap was removed and the excess packing buffer allowed to drain by gravity. The drained buffer was discarded and the gel was washed 3 times each with 500 µl deionized water. The column was centrifuged for 2 minutes at 1,000 xg to remove the excess water.

3- The column was placed in a clean 2.0 ml microcentrifuge tube and the hydroxylapatite peak fraction was applied carefully directly to the center of the column.

4- The column was centrifuged for 4 minutes at 1,000 xg.

The sample eluted from the desalting column was concentrated using a speed-vac and resolved together with the NE and the phosphocellulose peak through 10% SDS-PAGE. Unfortunately, we did not detect any band corresponding to PARP in the hydroxylapatite peak (Figure 8) after using the desalting column. There is a possibility that the desalting column retained PARP in addition to the potassium phosphate.

In order to clean the hydroxylapatite peak, the fraction was dialyzed against a buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, and 1 mM DTT for 3 hours at 4°C. The dialyzed fraction was centrifuged at 10,000 rpm for 5 minutes at 4°C and concentrated using a speed-vac.

The NE, phosphocellulose peak and the concentrated hydroxylapatite peak were resolved through 10% SDS-PAGE. PARP was detected by Western blot analysis using anti-human PARP antibody (1:500, BD PharMingen). A very clear band corresponding to PARP was detected in the hydroxylapatite peak (Figure 9). PARP was also highly enriched after this method of purification (Compare the density of PARP band in lane 3 with those in lanes 1 and 2, Figure 9).

The NE, Phosphocellulose peak, and the hydroxylapatite peak will also be resolved through 10% SDS polyacrylamide gels. The gel will be stained with silver or Coomassie blue stain to detect the purity of PARP with different purification steps.

III- PARP Activity in the Nuclear Fraction (NE) and in the Phosphocellulose Peak:

PARP assay was performed according to the methods of Jump and Smulson (1980), and Knights and Chambers (2001).

The standard reaction mixture (100 µl) contains 50 mM Tris-HCl, pH 8.0, 25 mM $MgCl_2$, 1 mM NAD^+, 10 µg activated DNA, and 1-2 µl ^{32}P-NAD (Perkin Elmer Life Sciences, specific activity 1000Ci/mmol) and increasing concentration of NE protein fractions. The reaction mixture was incubated at 25°C for 10 minutes. The poly(ADP-ribosyl)ated proteins were precipitated with 20% ice-cold trichloroacetic acid (TCA). The pellets were then washed with 10% TCA and dissolved in the scintillation fluid followed by liquid scintillation counting. The reactions were carried out in the absence or presence of 3-aminobenzamide (3-AB) which is a known inhibitor of PARP.

Figure 10A shows the increase in PARP activity with increasing concentration of proteins in the NE fraction. No activity was detected in the presence of 3-AB indicating that the measured activity is mainly due to PARP.

Since PARP needs single or double-stranded DNA breaks to be activated, we performed the assay in the absence or presence of activated DNA. As shown in Figure 10B, PARP was not active in the absence of activated DNA indicating that the activity of this enzyme is completely dependent on the presence of a damaged DNA.

PARP activity was also measured in the fractions eluted from phosphocellulose column. Figure 10C clearly demonstrates that most of the PARP activity was observed in the phosphocellulose peak (0.5 M NaCl eluted fraction).

We also compared PARP activity in the NE fraction and in the fractions eluted from phosphocellulose column. PARP activity was approximately doubled in the phosphocellulose peak relative to the NE fraction (Figure 10D, Compare PARP activity in **10 µg** NE protein fraction with that present in only **1 µg** phosphocellulose peak).

PARP activity will be also determined in the hydroxylapatite peak and the **specific activity** of PARP in that fraction will be compared with that in the NE fraction and phosphocellulose peak. The hydroxylapatite peak will be used to determine the Km and Vmax of PARP isolated from malignant and non-malignant breast cells.

IV- Two-Dimensional SDS-PAGE Analyses of PARP from Malignant and Non-Malignant Breast Cells:

In order to determine the isoelectric point (pI) and the 2-D PAGE mobility of PARP isolated from malignant and non-malignant breast cells, the nuclear extracts from MDA MB-468 and MCF-10A cells were prepared and analyzed using Bio-Rad Mini-Protean II tube cells (Bechtel et al., 1998). Approximately, 100 μg of protein were loaded onto the first dimension tube gel containing 9.2 M urea, 4% acrylamide, 20% Triton X-100, 1.6% Bio-Lyte 8/10 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte, 0.01% ammonium persulfate, and 0.1% TEMED. The proteins were separated along a pH gradient created using 100 mM NaOH (upper running buffer) and 10 mM H_3PO_4 (lower running buffer). The tube gel was placed onto a 10% SDS-polyacrylamide gel and the proteins were resolved by molecular weight. The proteins were transferred to a nitrocellulose membrane and PARP was detected using anti-human PARP antibody.

Since PARP is a basic protein with a pI value > 9.0, we could not separate PARP by this method. In order to solve this problem, we tried two alternatives:

1- Using nonequilibrium pH gradient electrophoresis (NEPHGE) according to the method of Anderson (1988) and Prasad et al. (1999). Proteins were loaded on the top of the tube gels and overlaid with 4 M urea to protect proteins from phosphoric acid, which was used in the upper reservoir. The lower reservoir was filled with NaOH. The electrical leads were reversed at the power supply and the gels were run at 400 V for 1 hour followed by 4 hours at 800 V. The tube gels were transferred to 10%SDS-PAGE for resolution in the second dimension.

2- Using the Bio-Rad IEF cell.
This cell performs the first dimension isoelectric focusing and can provide a maximum voltage of 10,000 V, which permits better focusing and resolution of the proteins. In addition, the precast immobilized pH gradient gel strips (Bio-Rad ReadyStrip IPG strips) were used in place of the first dimension tube gels. These strips provide reproducible gradients and eliminate the gradient drift that might occur with the tube gels.

PARP in the nuclear fractions have been analyzed using the IEF cell and IPG strips.

- Approximately 100 μg of cytosolic or nuclear fraction were desalted using the Bio-Spin 6 Tris Columns (Bio-Rad). The sample was concentrated using a SpeedVac.

- Prior isoelectric focusing, the IPG strips were rehydrated with a rehydration buffer containing 8 M urea, 1% CHAPS, 15 mM DTT, 0.1% Bio-Lyte 3/10 ampholyte, and 0.001% bromophenol blue.
- After SpeedVac, the protein sample was dissolved in 135 µl rehydration buffer and loaded in the sample loading well in the focusing tray. The IPG strip was placed into the channel tray by sliding it through the rehydration solution. After approximately 30 minutes, mineral oil was applied to each channel containing the IPG strip to prevent sample evaporation during the focusing step.
- Prior to running the second dimension, it was necessary to equilibrate the IPG strips. Two equilibration steps (15 minutes each) were necessary; the first step was required to saturate the strips with SDS and the reducing agent, while the second equilibration step was required to prevent protein re-oxidation during electrophoresis and alkylates the residual DTT to minimize vertical streaking.

Unfortunately, we were not able to detect PARP using either of the two alternatives. PARP isolated from MDA MB-468 were detected once and the methods were not reproducible.

We tried to re-use the Bio-Rad Mini-Protean II tube cells with some modifications. Instead of using 8/10 and 3/10 Bio-Lyte ampholytes (1.6 and 0.4%, respectively), we prepared the tube gels using **2% of 3/10 Bio-Lyte ampholyte**. By using this Bio-Lyte as the sole ampholyte in the tube gels, we were able to detect PARP in the NE fraction from both MDA MB-468 and MCF-10A cells. This method was reproducible and we obtained the same results by repeating the experiments more than 6 times.

Figure 11 clearly demonstrates that PARP from the breast cancer cells MDA MB-468 exhibits a different migration pattern, when resolved by 2D-SDS PAGE, as compared to the enzyme from non-malignant breast cells; indicating that this protein may be modified in breast cancer cells (Compare PARP migration in the upper and lower panels, Figure 11).

In order to check if this modification is due to poly(ADP-ribosyl)ation of the protein, the membrane was stripped off and re-probed with anti-PAR polyclonal antibody (1:1000, Trevigen) that recognizes the poly(ADP-ribose) polymer. The results indicated that PARP is not poly(ADP-ribosyl)ated in breast cancer cells (Figure 12) and the different migration pattern that PARP showed in breast cancer cells may be due to other chemical modification such as phosphorylation. Mass spectrometric analyses are underway to detect the nature of this modification.

V- Mass Spectrometric Analyses of PARP isolated from Non-Malignant and Malignant Breast Cells

PARP purified from malignant and non-malignant breast cells will be resolved using 10% SDS-PAGE. The gel will be stained by using either silver or Coomasie stains. The method is based on that of Shevchenko et al. (1996) and it includes reduction and acetamidation steps according the following protocol:

1- The bands corresponding to PARP will be excised from silver or Coomassie-stained gel. The gels band will be cut into small pieces and transferred to eppendorff tubes.
2- 25-35 µl acetonitrile will be added to cover the gel pieces and will be incubated for 10 minutes at room temperature to dehydrate and shrink gel pieces.
3- Acetonitrile will be removed. Speed-vac to dryness.
4- The gel particles will be swollen by incubation in 150 µl 10 mM DTT in 100 mM NH_4HCO_3 for 1 hour at 56°C.
5- The tubes will be cooled to room temperature and the DTT solution will be replaced with 150 µl 55 mM iodoacetamide in 100 mM NH_4HCO_3. The tubes will be incubated for 45 minutes at room temperature in dark with occasional vortexing.
6- The solution will be removed and the gel pieces will be washed with 150 µl 100 mM NH_4HCO_3 and incubated for 10 minutes at room temperature.
7- NH_4HCO_3 will be replaced with 150 µl acetonitrile to dehydrate the gel pieces and will be incubated for 10 minutes at room temperature.
8- Washing steps 6 and 7 will be repeated and the acetonitrile will be removed. The gel pieces will be dried using a speed-vac.
9- The gel pieces will be incubated in ice water bath for 45 minutes and will be allowed to swell in 25-35 µl digestion buffer.
   Digestion buffer consists of 12.5 ng/µl trypsin (Promega sequence grade modified porcine trypsin) in 50 mM NH_4HCO_3.
10- The trypsin-containing buffer will be removed and 5-10 µl NH_4HCO_3 without trypsin to keep gel pieces wet during cleavage at 37°C overnight.
11- The tubes will be centrifuged for 1 minute and the supernatant will be saved in a PCR tube.
12- 20 µl 20 mM NH_4HCO_3 will be added to cover the gel pieces and will be incubated for 10 minutes at room temperature. The supernatant will be transferred to the PCR tube from step # 11,
13- 25 µl 5% formic acid/50% acetonitrile will be added to the gel pieces and incubated at room temperature for 20 minutes.
14- Spin at 14,000 rpm for 1 minute. The formic acid/acetonitrile solution will be removed and saved in the same PCR tube from step 11.
15- Formic acid extraction (steps 13-14) will be repeated twice more.
16- The PCR tubes will be dried in speed-vac to complete dryness and stored at -20°C till analysis.

KEY RESEARCH ACCOMPLISHMENTS:

- Phosphocellulose and hydroxylapatite purification of PARP from malignant and non-malignant breast cells (MDA MB-468 and MCF-10A, respectively).

- Combining successfully the phosphocellulose and hydroxylapatite chromatography techniques to purify and enrich PARP from malignant and non-malignant breast cells. This step is considered the key step for determining and comparing the kinetic and physical properties of the enzyme isolated from the two cell lines.

- Analysis of PARP protein from malignant and non-malignant breast cells using 2D-SDS PAGE.

- Detection of a modified form of PARP in breast cancer cells.

- Setting up the *in vitro* PARP activity assay as an important step towards studying the kinetics of the enzyme in both kinds of breast cells.

REPORTABLE OUTCOMES:

Abstracts:

1- Abdel-Aziz W, Han S, Hickey RJ, and Malkas LH (2002). Purification and functional characterization of breast tumor cell poly(ADP-ribose) polymerase. Presented at the *Era of Hope* department of Defense Breast Cancer Research Meeting, Orlando, FL, September 25-28.

2- Abdel-Aziz W, Hoelz D, Malkas LH, and Hickey RJ (2002). Purification and functional characterization of breast cancer cell poly(ADP-ribose) polymerase. Presented at the 25th Annual San Antonio Breast Cancer Symposium, San Antonio, TX, December 11-14.

3- Abdel-Aziz W, Malkas LH, Hoelz D, and Hickey RJ (2003). Proteomic analysis of breast cancer cell poly(ADP-ribose) polymerase. Presented at the 94th AACR Annual Meeting, Washington DC, July 11-14.

4- Abdel-Aziz W, Malkas L, Hoelz D, and Hickey R (2003). The molecular "nick sensor" protein poly(ADP-ribose) polymerase is modified in breast cancer cells. Submitted for presentation at the AACR-NCI-EORTC International Conference on *Molecular Targets and Cancer Therapeutics*, Boston, MA, November 17-21.

Research Articles:

- Abdel-Aziz W, Malkas LH, Hoelz D, and Hickey RJ (2003). Proteomic and functional analysis of breast cell poly(ADP-ribose) polymerase. In preparation.

CONCLUSIONS:

- PARP is confirmed by SDS-PAGE and mass spectrometric analyses to be one of the components of the DNA synthesome.

- PARP has been successfully purified from malignant and non-malignant breast cells using phosphocellulose chromatography. Most of PARP protein was eluted from the phosphocellulose column with 0.5 M NaCl.

- PARP enzymatic activity was highly enriched after this step of purification.

- PARP was further concentrated and purified from the 50-kDa proteins by filtration through Millipore Centrifugal filters. This step of purification resulted in further enrichment of PARP.

- The combination of the hydroxylapatite with phosphocellulose chromatography techniques resulted in further purification and enrichment of PARP. The phosphocellulose peak was applied onto hydroxylapatite matrix and most of PARP protein was detected in the 500 mM potassium phosphate eluted fraction.

- Two-dimensional SDS-PAGE analysis of PARP indicated the presence of a modified form of this protein in breast cancer cells.

- Mass spectrometric analysis of PARP isolated from non-malignant and malignant breast cells are underway to identify the nature of this modification. In addition kinetic and physical properties determination of PARP from the two cell lines are in progress to explain how PARP modification may contribute to the observed decrease in replication fidelity in breast cancer cells.

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APPENDIX COVER SHEET

FIGURES 1-12

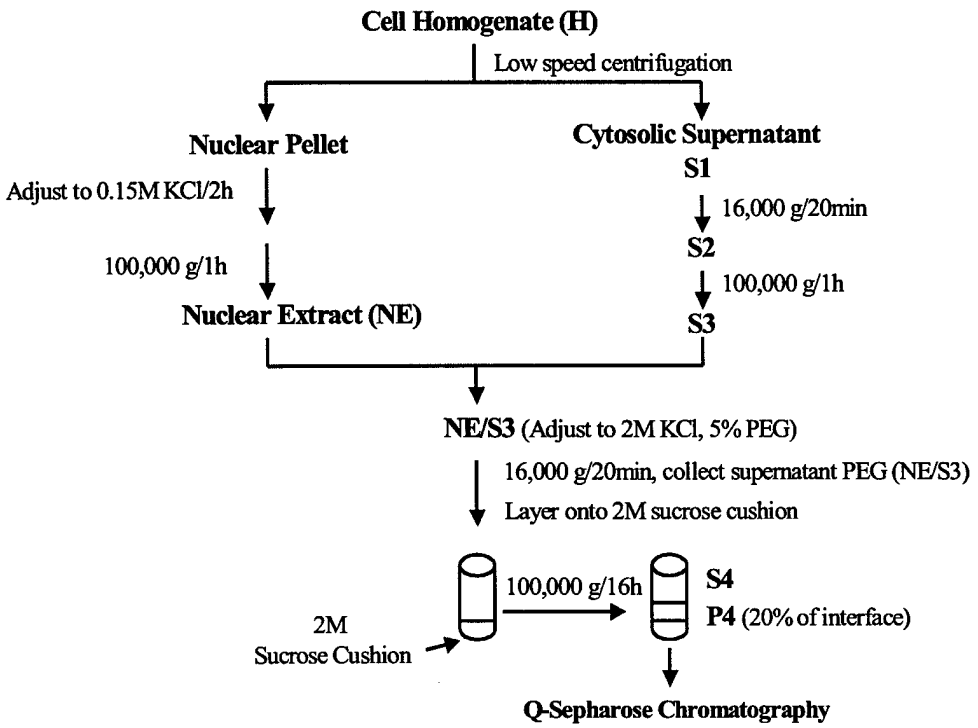

Figure 1A: Flow diagram of subcellular fractionation scheme

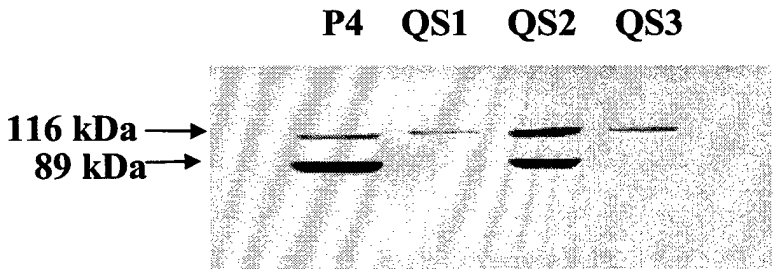

Figure 1B: Immunoblot analysis of PARP in P4 and Q-Sepharose fractions.

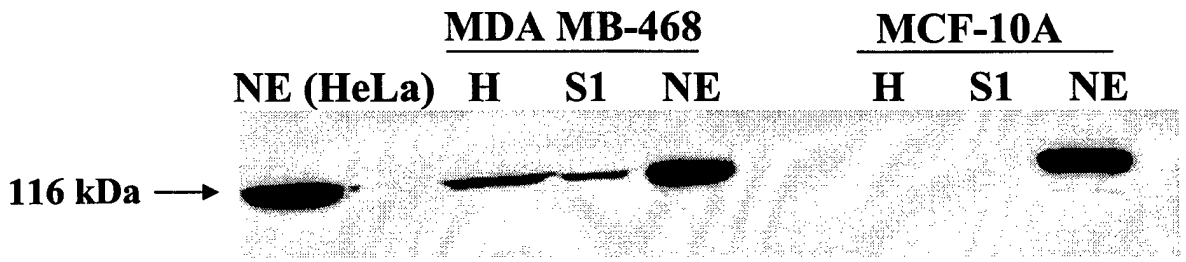

Figure2: Immunoblot analysis of PARP in H, S1, and NE fractions from MDA MB-468 and MCF-10A cells. 50μg proteins from H, S1, and NE fractions were resolved through 10% SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-PARP monoclonal antibody (BD PharMingen, 1:500 dilution). NE fraction from HeLa cells were resolved in a parallel lane and was used a positive control.

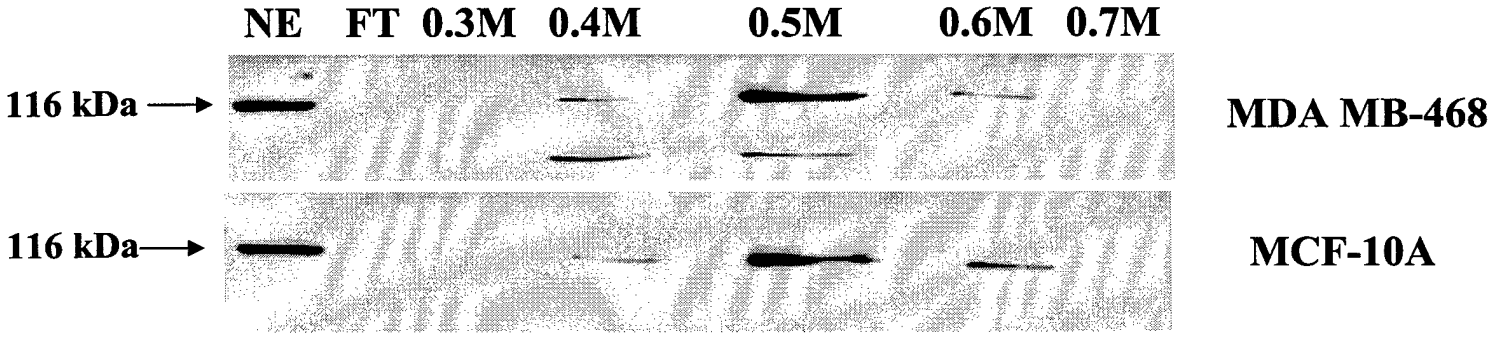

Figure 3: Immunoblot analysis of PARP in the NE, flow throw (FT), and in the fractions eluted from phosphocellulose column with increasing concentration of NaCl (0.3-0.7M).

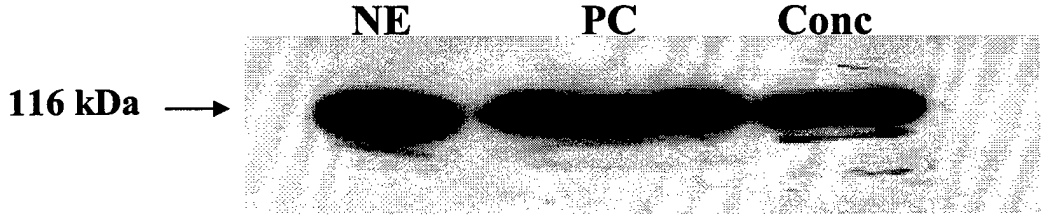

Figure 4: Immunoblot analysis of PARP in the NE fraction, phosphocellulose peak (fraction eluted with 0.5 M NaCl; PC) and after concentration of the later fraction using Microcon Centrifugal Devices.

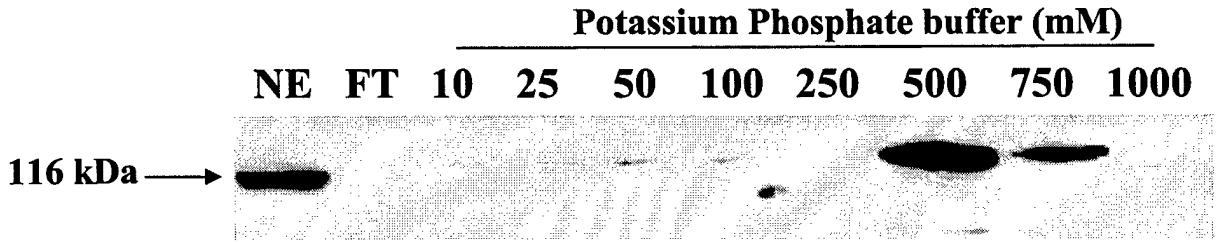

Figure 5: Immunoblot analysis of PARP in the NE, Flow Throw (FT), and in the fractions eluted from hydroxylapatite column with increasing concentrations of potassium phosphate (10-1000 mM).

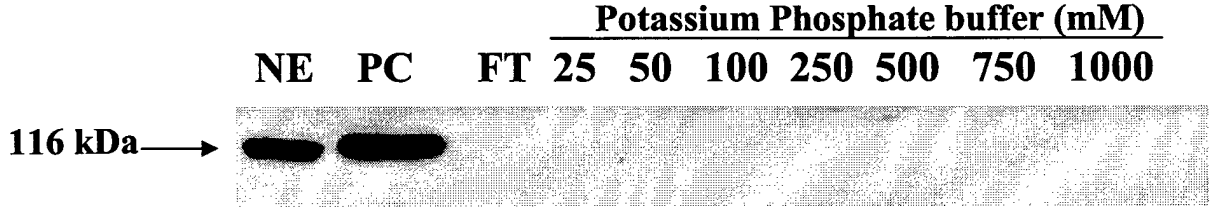

Figure 6: Immunoblot analysis of PARP in the NE, phosphocellulose peak (PC), FT, and in the fractions eluted from hydroxylapatite column with a stepwise increasing concentration of potassium phosphate (25-1000 mM).

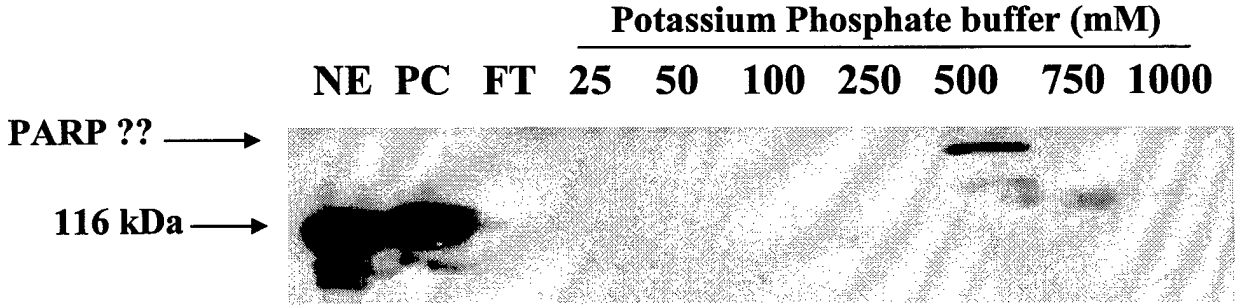

Figure 7: Immunoblot analysis of PARP in the NE, PC, FT, and in the fractions eluted from hydroxylapatite column after concentrations using a speed-vac.

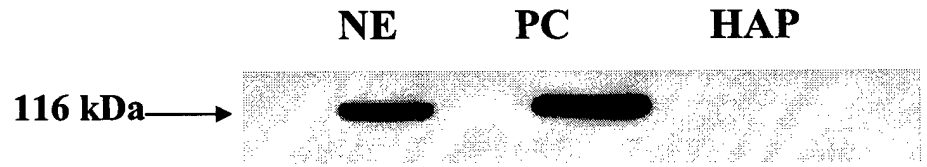

Figure 8: Immunoblot analysis of PARP in NE, PC, and hydroxylapatite peak (HAP; Fraction eluted with 500 mM potassium phosphate) after applying the HAP onto the BioRad desalting column and speed-vac.

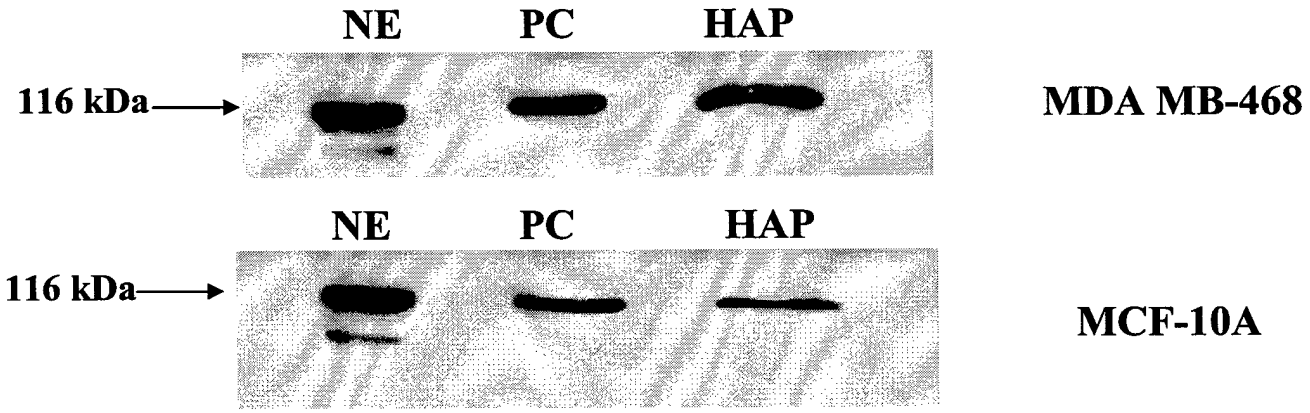

Figure 9: Immunoblot analysis of PARP in NE, PC, and HAP after dialysis of the later fraction for 3 hours at 4°C, with 2 changes of the buffer followed by speed-vac.

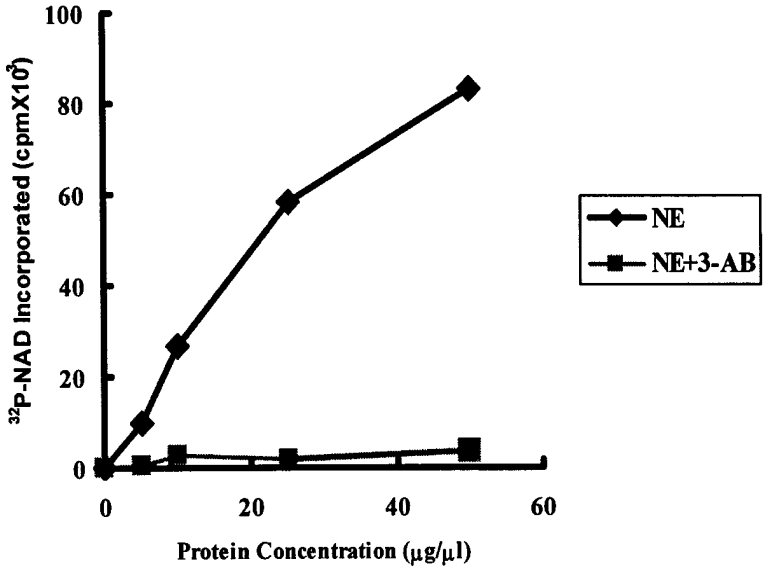

Figure 10A: PARP activity in NE fraction in the absence of presence of 3-AB.

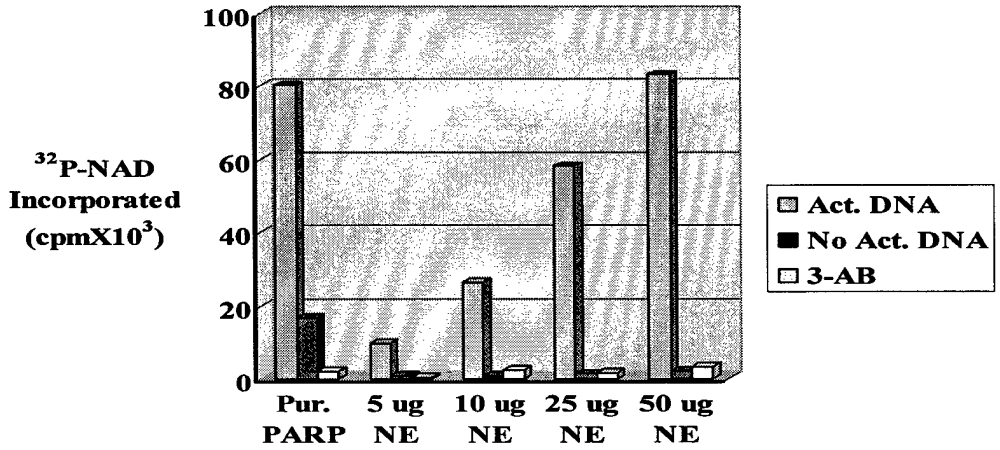

Figure 10B: PARP activity in NE fraction isolated from breast cancer cells (MDA MB-468) in the absence or presence of activated DNA and in the absence or presence of 3-AB. The activity of the enzyme is completely dependent on the presence of DNA strand breaks and is inhibited in the presence of 3-AB. The same experiment was carried out using 20 μg of partially purified enzyme (Trevigen) for comparison.

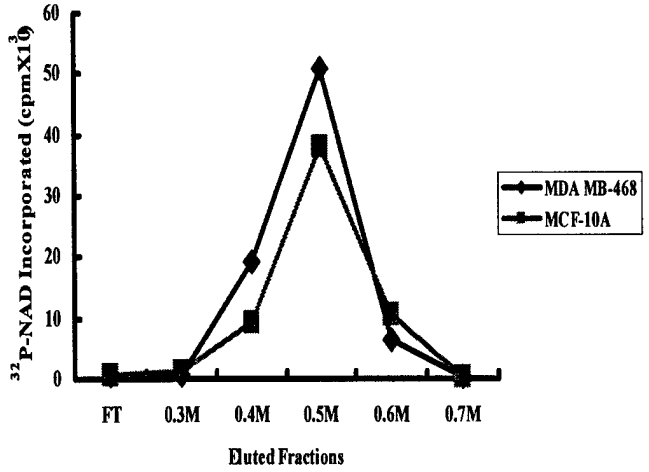

Figure 10C: PARP activity in the FT and in fractions eluted fror phosphocellulose with increasing concentrations of NaCl.

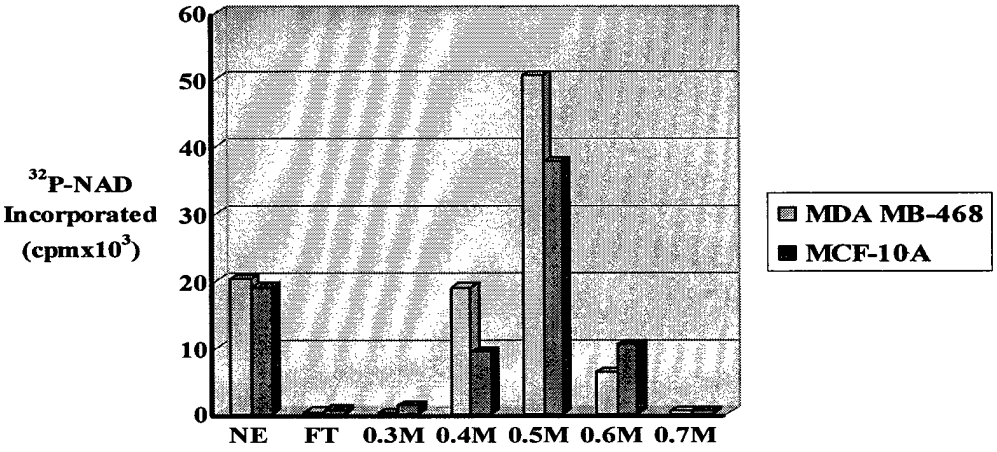

Figure 10D: Enrichment of PARP enzymatic activity after its partial Purification using phosphocellulose chromatography.

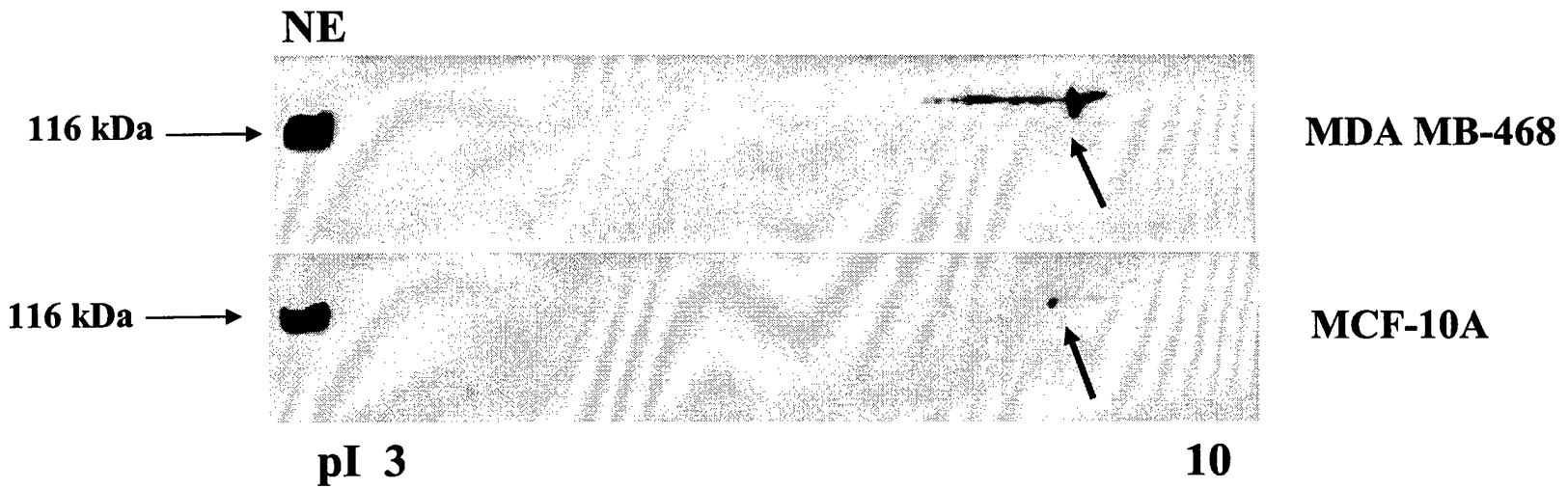

Figure 11: 2D SDS-PASGE analysis of PARP in MDA MB-468 and MCF-10A cells. 100µg of NE protein were loaded onto the first dimension tube gel and the proteins were separated along a pH gradient created using 100 mM NaOH and H_3PO4. The tube gels were placed onto 10% SDS-polyacrylamide gels and PARP was detected by Western blot analysis. 50 µg NE protein fraction were resolved in the protein marker lane, in the second dimension gels, and were used as positive controls.

Figure 12: The modified form of PARP present in malignant breast cells is not poly(ADP-ribosyl)ated. The membrane from Figure 11 was stripped off and reprobed with anti-PAR polyclonal antibody (Trevigen, 1:1000) that recognizes the poly(ADP-ribose) polymer.